Acceleration of Proliferative Response of Mouse Fibroblasts by Short-Time Pretreatment with Polyphenols

博士論文

Acceleration of Proliferative Response of Mouse Fibroblasts by Short-Time Pretreatment with Polyphenols

（ポリフェノール短時間前処理によるマウス線維芽細胞の増殖反応促進）

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Content

ABSTRACT

Under the hypothesis that photo-irradiated proanthocyanidin could accelerate wound healing through reactive oxygen species (ROS) formation, the effect of proanthocyanidin on 3T3-L1 mouse fibroblasts was examined with or without photo-irradiation. As a result, irrespective of presence or absence of photo-irradiation, only 1 min exposure of the cells to proanthocyanidin resulted in accelerated proliferation of the cells in a concentration-dependent manner. Similarly to proanthocyanidin, 1 min pretreatment with catechin, caffeic acid and chlorogenic acid accelerated the proliferative response, but gallic acid, epicatechin gallate, epigallocatechin, and epigallocatechin gallate failed. If incorporated active ingredient such as proanthocyanidin for such a short time as 1 min accelerates the proliferation response, a bioassay was conducted by utilizing antioxidant potential of proanthocyanidin. That is, intracellular oxidation of 2',7'-dichlorodihydrofluorescin induced by H_2O_2 was significantly inhibited when the cells were pretreated with proanthocyanidin for 1 min, suggesting that incorporated proanthocyanidin into the cells exerted antioxidant effect. This was also supported by a liquid chromatography/mass spectrometry analysis in which incorporation of proanthocyanidin components such as catechin monomers and dimers into the cells within 1 min was confirmed. These results suggest that active polyphenolic compounds such as proanthocyanidin, catechin, caffeic acid, and chlorogenic acid incorporated into the cells in such a short time as 1 min could accelerate the proliferative response of the cells.

Introduction

In our previous study where the acute locally injurious property of hydroxyl radical generation system by photolysis of hydrogen peroxide (H_2O_2) was examined using a rat excisional skin wound model [1], single topical treatment with the technique for a short time as 2 min tended to accelerate the wound healing process toward wound closure as indicated by the time course measurement of wound areas. Recent *in vitro* and *in vivo* observations suggest that reactive oxygen species (ROS), and mainly H_2O_2, interfere with cell signaling acting like a second messenger and inducing adaptive responses, and this is particularly observed in skin wound healing where cells are exposed to H_2O_2 following injury [2-5]. Therefore, it is suggested that the disinfection technique would have a healing effect likely attributable to H_2O_2.

It has also been reported recently that photo-irradiated polyphenols such as gallic acid and proanthocyanidin, which consists of a monomer and a polymer of flavan-3-ol, such as catechin, epicatechin, and epicatechin gallate, with an average degree of polymerization between 2 and 17 [6, 7], exerts potent bactericidal activity via ROS formation including H_2O_2 [8, 9]. The cue of our previous studies on bactericidal activity of photo-irradiated polyphenols came from the idea that polyphenolic hydroxyl groups of gallic acid or proanthocyanidin are oxidized by the photo-irradiation, and a resultant proton coupled with an electron transfers to dissolved oxygen resulting in H_2O_2 generation as reported previously [10, 11]. Then, the resultant H_2O_2 would be photolyzed to generate hydroxyl radicals, which in turn would cause oxidative damage, including lipid peroxidation to the bacterial cells as reported in our previous studies [8, 12]. Based on these findings, the hypothesis that photo-irradiated polyphenols could accelerate wound healing through ROS formation was made. If photo-irradiated polyphenols are

proven to be effective on wound healing, they would be a novel therapeutic technique having an ability not only to accelerate wound healing but to kill bacteria effectively. In other words they would be a novel antiseptic wound healing technique. Fibroblast proliferation is one of the pivotal events in the wound healing process [13-15], so that the effect of short-time treatment with proanthocyanidin on the proliferative response of fibroblasts was firstly examined with or without photo-irradiation followed by examining the effect of various kinds of polyphenols. The schematic illustration for the hypothesis in relation to *in vitro* fibroblast proliferation is shown in Fig. 1.

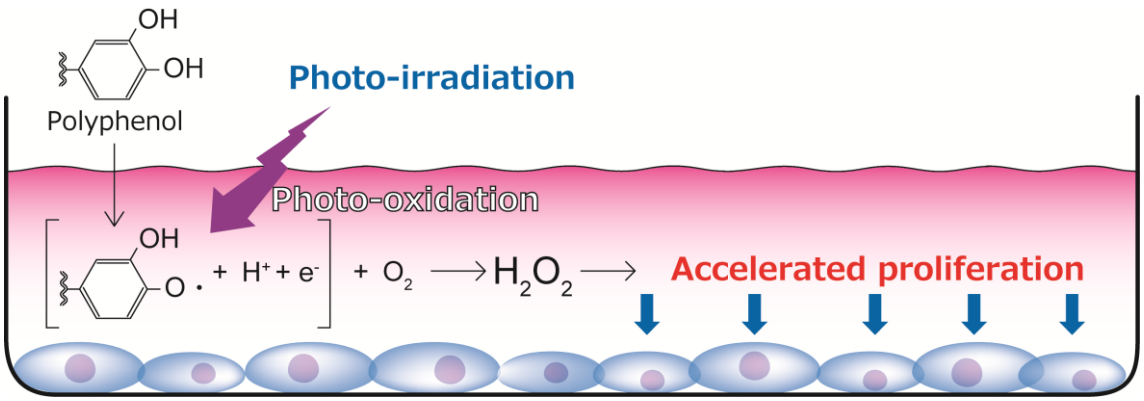

Figure 1

Schematic illustration for the hypothesis that photo-irradiated polyphenols could accelerate fibroblast proliferation through H_2O_2 formation

Materials and Methods

Test substances

Polyphenols were purchased from the following sources: proanthocyanidin (Leucoselect® that contains a mixture of monomers and polymers of flavan-3-ol) from Indena (Milano, Italy); gallic acid and caffeic acid from Tokyo Chemical Industry (Tokyo, Japan); chlorogenic acid, (+)-catechin, (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin gallate (EGCG) from Sigma-Aldrich (St. Louis, MO, USA). Chemical structures of monomeric polyphenols tested in the present study are shown in Fig. 2. Each test substance was dissolved in physiological saline followed by filter sterilization. In the present study, designated concentrations of each polyphenol were prepared on a weight basis but not on a molecular-weight basis. Since proanthocyanidin contains a monomer and a polymer of flavan-3-ol, such as catechin, (-)-epicatechin (EC), and ECG, so that proanthocyanidin solutions could not be prepared on a molecular-weight basis. Thus, other polyphenol solutions were also prepared on a weight basis.

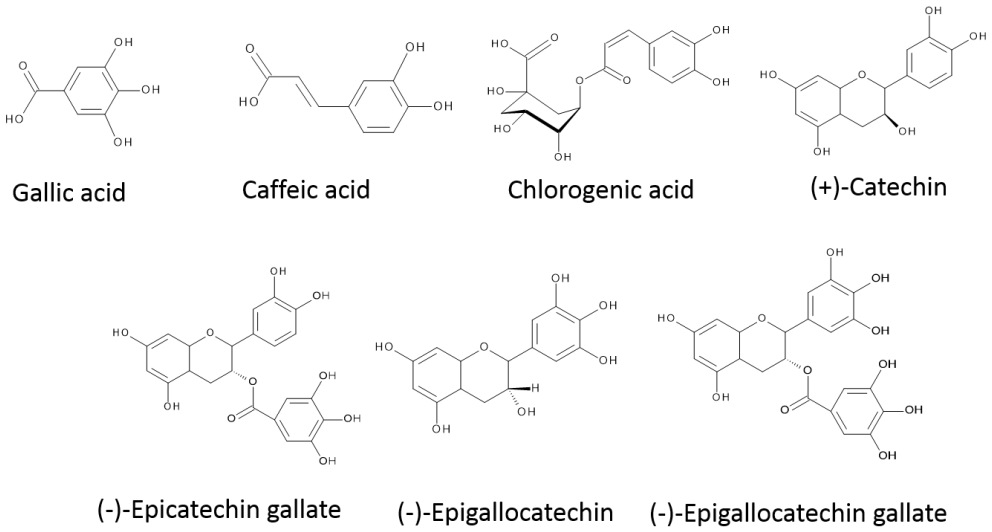

Figure 2

Chemical structures of gallic acid, caffeic acid, chlorogenic acid, and catechins tested

Cell culture and cell proliferation assay

3T3-L1 mouse fibroblasts were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific), 100 U/ml of penicillin (Wako Pure Chemicals), and 0.1 mg/ml of streptomycin (Wako Pure Chemicals) was used as a medium for cell culture. An aliquot (100 μl) of the cell suspension (2 x 10^4 cells/ml) was placed in each well of a 96 well-culture plate. The plates were incubated at 37℃ in humidified 5% CO_2 for 24-28 h for sub-confluent (20-40%) condition. The medium was then replaced with a sterile physiological saline or test substance solutions. After cells were exposed to test substance for 1 min or longer, the cells were washed and placed in fresh medium, and were further incubated for 24-28 h to determine cell viability by the methyl thiazolyl tetrazorium (MTT) assay in which an insoluble formazan converted from MTT was colorimetrically determined at 595 nm by using a microplate reader (FilterMax F5, Molecular Devices, Sunnyvale, CA, USA). The MTT assay was performed by using a kit (TACS® MTT Cell proliferation Assay, Trevigen Inc., Gaithersburg, MD, USA). In a study where the cells were exposed to photo-irradiation, a continuous-wave laser device (RV-1000; Ricoh Optical Industries, Hanamaki, Japan) was used as a light source that irradiates the light at wavelength of 405 nm ± 5 nm and an output power from an indium gallium nitride laser diode was set at 300 mW. The diameter of the irradiation field was set to equal to that of the well (6.4 mm) so that almost all of the light could pass through the test solution. Thus, the energy density was calculated to be 930 mW/cm^2. Besides the MTT assay, the neutral red (NR, Wako Pure Chemical Industries, Osaka, Japan) uptake assay was also conducted to

evaluate cell viability in a proanthocyanidin experiment. In brief, the medium was replaced with the medium containing 150 μg/ml of NR followed by further incubation for 3 h. Then NR was extracted with 50% ethanol containing 1% acetic acid for colorimetric determination at 540 nm by using the microplate reader (FilterMax F5). The MTT assay is a quantitative colorimetric assay to quantify mitochondrial activity [16, 17], thus it detects living cells only and the colorimetric endpoint generated is directly proportional to the number of viable cells. The NR assay is based on the *in vitro* incorporation of NR into lysosomes of living mammalian cells [18, 19]. In the present paper, cell proliferation was basically determined by the MTT assay unless a specified description. Schematic illustration for the basic experimental schedule for 1 min pretreatment is shown in Fig. 3. In the experiments, the maximum concentration of polyphenols except proanthocyanidin was set to be 0.5 mg/ml because some polyphenols such as (+)-catechin and caffeic acid were hard to be dissolved at higher concentrations (*e.g.*, 1 mg/ml).

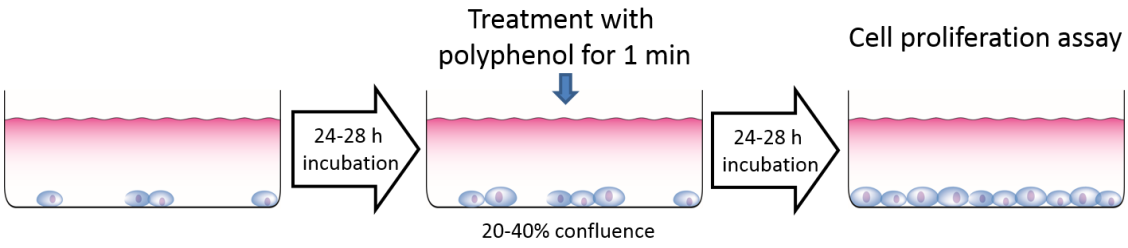

Figure 3

Schematic illustration for the basic design to examine the effect of 1 min pretreatment with polyphenol on the proliferative response of mouse fibroblasts

H_2O_2-load onto the cells and detection of intracellular oxidative stress

Intracellular oxidative stress caused by H_2O_2-load was determined by using a kit (Oxiselect^{TM} Intracellular ROS Assay Kit, Cell Biolabs, Inc., San Diego, CA, USA) for intracellular ROS assay. In this assay, a cell-permeable probe, 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) diffuses into cells and is deacetylated to a nonfluorescent product, 2',7'-dichlorodihydrofluorescin (DCFH) by cellular esterases. In the presence of cytosolic ROS, DCFH is oxidized to a highly fluorescent, 2',7'-dichlorodihydrofluorescein (DCF).

Cells were similarly cultured for four days to achieve full confluence. Then 100 μl of 1 mM DCFH-DA dissolved in serum-free medium was added to each well, and incubated at 37℃ for 60 min. After washing the cells twice with phosphate buffered saline (PBS, pH 7.4), the cells were exposed to a sterile physiological saline or 1 mg/ml of proanthocyanidin dissolved in the physiological saline for 1 min. After washing the cells twice with PBS, 100 μl of 1 mM H_2O_2 prepared in serum-free medium was added to each well, and incubated at 37℃ for 20 min. After washing the cells twice with PBS, 100 μl of serum-free medium and 100 μl of 2X Cell Lysis Buffer were added to each well, followed by mixing thoroughly and incubation for 5 min. After transferring 150 μl of the mixture to each well of a black 96-well culture plate, fluorescence was read at the excitation and emission wavelengths of 485 and 535 nm by using the microplate reader (FilterMax F5). Designated concentrations of DCF were used for the standard curve, and the magnitude of oxidative stress was expressed as DCF equivalent (nM).

Liquid chromatography/mass spectrometry (LC/MS) analysis of proanthocyanidin in the cells

An aliquot (1 ml) of the cell suspension (2 x 10^4 cells/ml) was placed in each well of a 12 well-culture plate. The plates were similarly incubated for four days to achieve full confluence. The medium was then replaced with a sterile physiological saline or 1 mg/ml of proanthocyanidin dissolved in the physiological saline, and kept for 1 min. After washing the cells twice with PBS, 0.2 ml of a cell lysis buffer (Glo Lysis Buffer, Promega Corp., Madison, WI, USA) was added to each well, and kept for 5 min. Then, 0.8 ml of ethanol was added to each well followed by mixing. The mixture was transferred to a test tube and centrifuged at 1200 x g for 20 min. The supernatant was then concentrated 10 times by using a centrifugal concentrator (CC-181, Tomy Seiko Co., Ltd., Tokyo, Japan). Following filtration through a 0.2 μm PVDF filter, the resultant sample was injected into the electrospray ion source of a QSTAR Elite ESI quadrupole time-of-flight (Q-TOF) MS instrument (AB Sciex, Framingham, MA, USA) coupled to Agilent 1200 series (Agilent technologies, Santa Clara, CA, USA). Chromatographic separation was performed on an Inertsil ODS-4 (2.0×250 mm, GL Sciences Inc., Tokyo, Japan) at 40°C. As for gradient elution, solvent A was water with 0.1% formic acid, and B was methanol with 0.1% formic acid. The gradient elution was programmed as follows: 0-30 min, 5-100% B. The flow rate was 0.5 ml/min, the injection volume was 5 μl, and UV detection was carried out by photodiode array detector. ESI-MS spectra were recorded for 30 min in the *m/z* region from 100 to 2,000 Da with the following instrument parameters: ion spray voltage = 5,500 V, source gas = 50 l/min, curtain gas = 30 l/min, declustering potential = 50V, focusing potential = 250 V, temperature = 450°C, and detector voltage = 2,300 V. LC/MS

analysis was performed by high resolution ESI-MS (HRESIMS; R $\geqq$ 10,000, the tolerance for mass accuracy is 10 ppm).

Statistical analysis

Statistical significances ($p<0.05$) in viable cells as expressed as % of control were assessed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test or Tukey-Kramer HSD comparison test.

Results

Effect of short-time exposure of mouse fibroblasts to proanthocyanidin with or without photo-irradiation on the proliferative response

Figure 4 shows the proliferative response of the cells determined by the MTT assay 24 h after the treatment with proanthocyanidin for 1 min with or without photo-irradiation. Irrespective of with or without photo-irradiation, pretreatment with proanthocyanidin for 1 min significantly accelerated the proliferative response of the cells in a concentration dependent manner although the proliferative response of the cells with photo-irradiation was slightly suppressed in whole as compared with that without photo-irradiation. To further confirm the effect of 1 min pretreatment with proanthocyanidin, the proliferative response of the cells was determined by the NR uptake assay (Fig. 5). Similarly to the case with the MTT assay, accelerated proliferative response was observed in the cells pretreated with proanthocyanidin for 1 min.

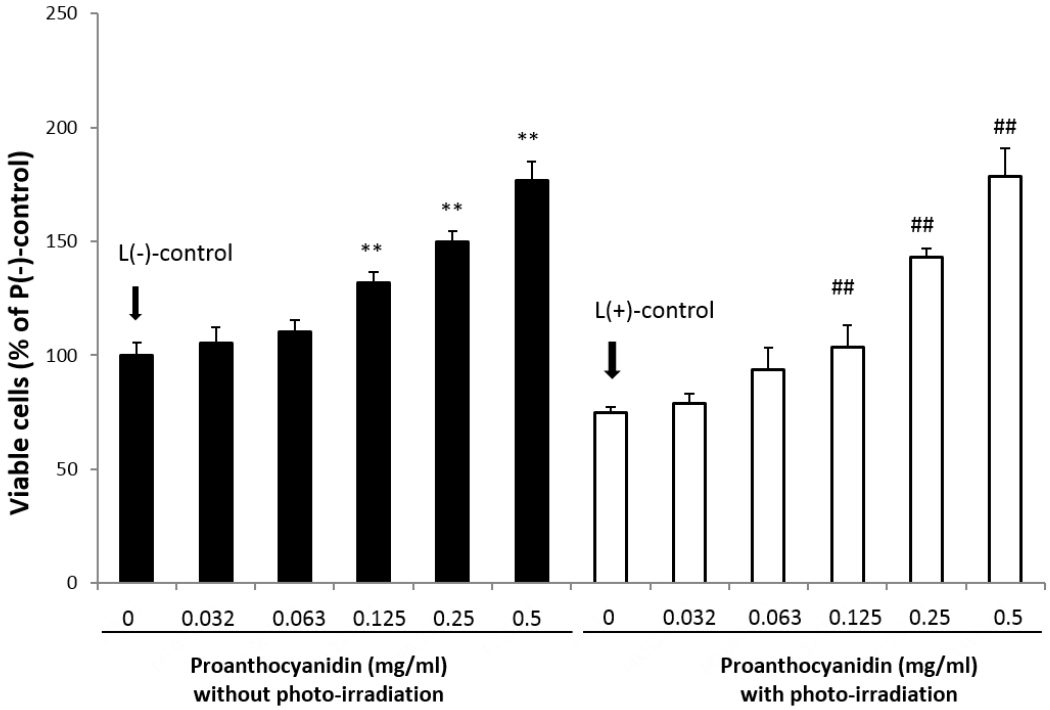

Figure 4

Effect of 1 min pretreatment with different concentrations of proanthocyanidin with or without photo-irradiation on the proliferative response of mouse 3T3-L1 fibroblasts

Each value represents the mean + standard deviation (n=3).

Significant differences from the corresponding controls are shown as ** $p<0.01$ and ## $p<0.01$ (Dunnett's multiple comparison test).

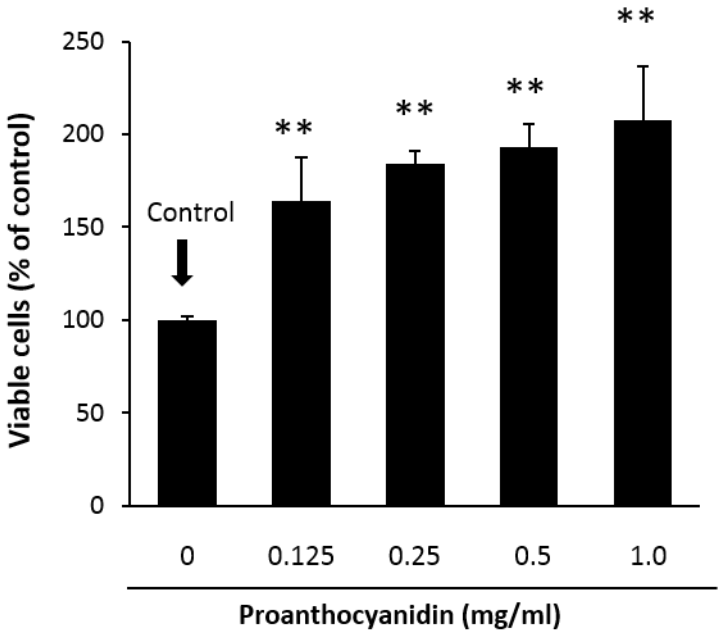

Figure 5

Effect of 1 min pretreatment with different concentrations of proanthocyanidin on the proliferative response of mouse 3T3-L1 fibroblasts determined by the neutral red uptake assay

Each value represents the mean + standard deviation (n=3).

Significant differences from the control are shown as ** $p<0.01$ (Dunnett's multiple comparison test).

Effect of short-time exposure of mouse fibroblasts to monomeric polyphenol compounds (gallic acid, caffeic acid, chlorogenic acid, and catechin) on the proliferative response

Figure 6 shows the proliferative response of the cells 24 h after the treatment with the phenolic compounds (gallic acid, caffeic acid, chlorogenic acid, and catechin) for 1 min. Pretreatment with caffeic acid, chlorogenic acid, and catechin for 1 min accelerated the proliferative response of the cells in a concentration dependent manner, but gallic acid failed to accelerate the proliferation.

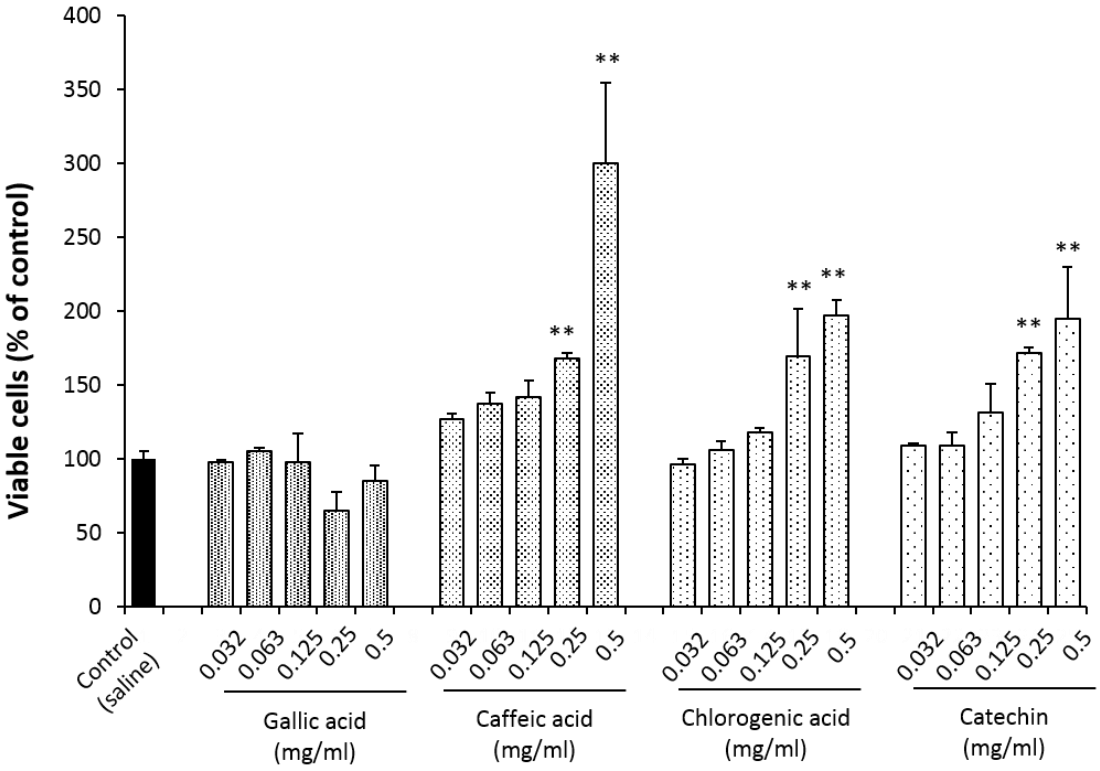

Figure 6

Effect of 1 min pretreatment with different concentrations of gallic acid, caffeic acid, chlorogenic acid, and (+)-catechin on the proliferative response of mouse 3T3-L1 fibroblasts

Each value represents the mean + standard deviation (n=3).

Significant differences from the control are shown as ** $p<0.01$ (Dunnett's multiple comparison test).

Effect of short-time exposure of mouse fibroblasts to gallated catechins and EGC on the proliferative response

Figure 7 shows the proliferative response of the cells 24 h after the treatment with the gallated catechins (ECG and EGCG) and EGC for 1 min. Pretreatment with any of the catechins tested did not accelerate the proliferative response of the cells when the catechins were treated within a range of concentrations (0.032 to 0.5 mg/ml).

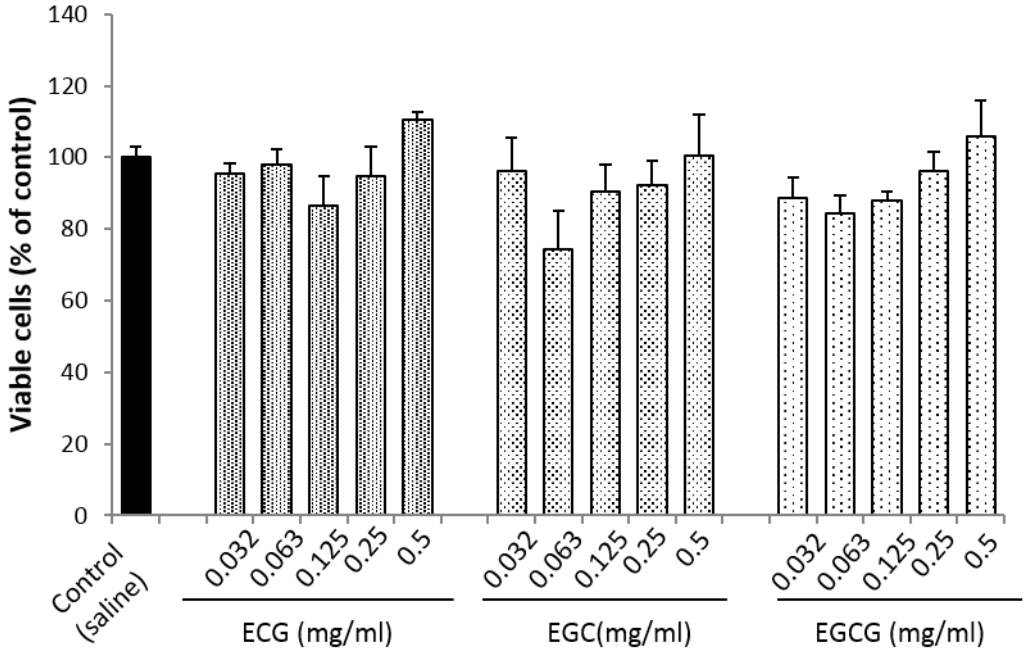

Figure 7

Effect of 1 min pretreatment with different concentrations of (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin gallate (EGCG) on the proliferative response of mouse 3T3-L1 fibroblasts

Each value represents the mean + standard deviation (n=3).

Effect of exposure time of proanthocyanidin and caffeic acid on the proliferative response of mouse fibroblasts

The proliferative responses of the cells pretreated with proanthocyanidin (0.5 mg/ml) and caffeic acid (0.5 mg/ml) for 0.5-32 min are summarized in Fig. 8. In the case of proanthocyanidin, even the 0.5-min pretreatment significantly accelerated the response, and this acceleration was similarly observed under the 32-min pretreatment condition. In contrast, although pretreatment with caffeic acid for 0.5-2 min resulted in significant acceleration of the proliferative response, the response was reduced to the control level in the pretreatment for 4-16 min, and to below the control level in the 32-min pretreatment.

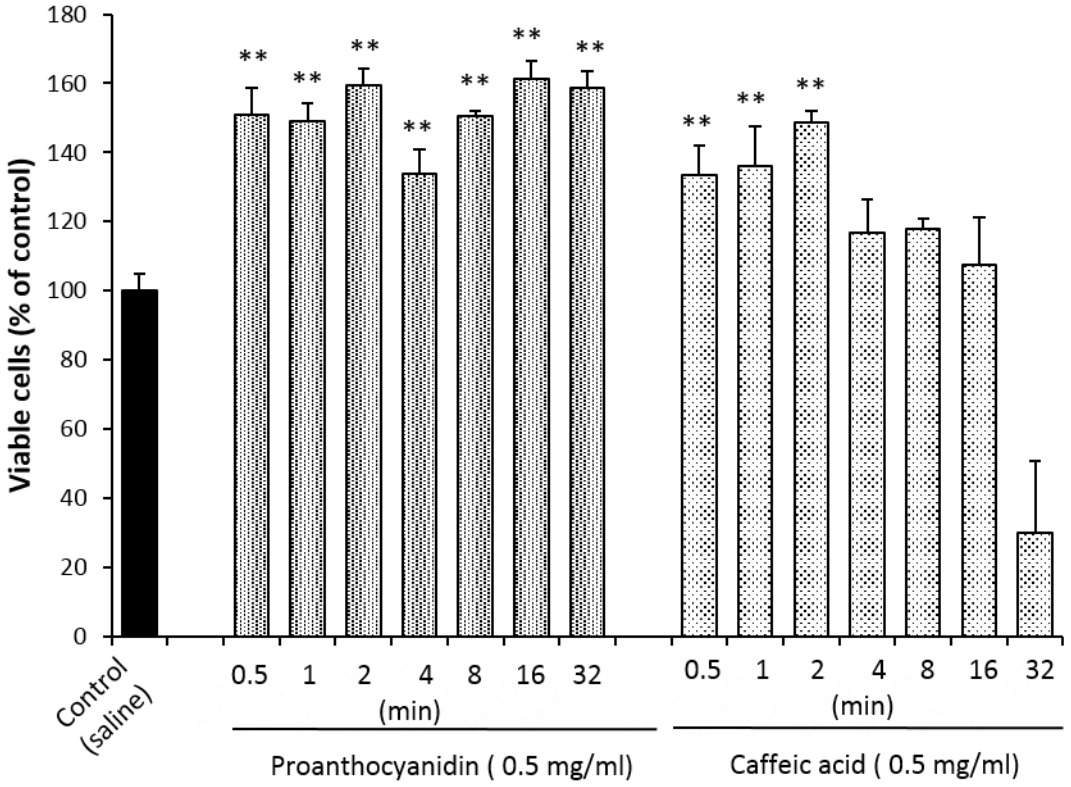

Figure 8

Effect of pretreatment time with proanthocyanidin (0.5 mg/ml) and caffeic acid (0.5 mg/ml) on the proliferative response of mouse 3T3-L1 fibroblasts

Each value represents the mean + standard deviation (n=3).

Significant differences from the control are shown as ** $p<0.01$ (Dunnett's multiple comparison test).

H_2O_2-induced oxidative stress

H_2O_2-induced oxidative stress as expressed as DCF equivalent, which indicated the magnitude of oxidation of DCFH caused by H_2O_2-load, is summarized in Fig. 9. The amount of intracellular DCF formed by H_2O_2-load significantly increased to approximately 150% in the cells treated with 1 mM H_2O_2 when compared to that in the control group (saline). The increase in DCF induced by H_2O_2 was significantly suppressed to less than the control level when the cells were pretreated with 1 mg/ml of proanthocyanidin for 1 min.

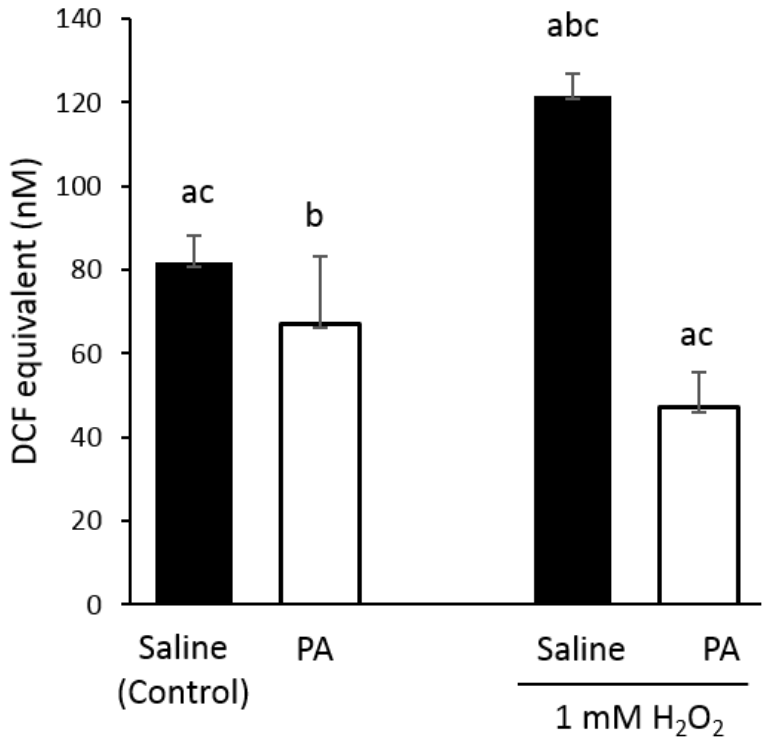

Figure 9

Effect of pretreatment with proanthocyanidin (PA, 1 mg/ml) for 1 min on intracellular oxidative stress caused by H_2O_2-load

Since fluorescent 2’7’-dichlorodihydrofluorescin (DCF) was used as a standard, magnitude of oxidative stress is expressed as DCF equivalent (nM).

Each value represents the mean + standard deviation (n=4).

Significant differences ($p<0.05$) between the two groups are demonstrated by the same alphabet (Tukey-Kramer HSD multiple comparison test).

LC/MS analysis of proanthocyanidin in mouse fibroblasts

According to the manufacture (Indena) of the proanthocyanidin used in the present study, the content of proanthocyanidin was 95-105% indicating that almost no compounds other than catechin monomers and oligomers were contained. Based on this information, the ESI mass spectrum suggested that the proanthocyanidin standard was at least consisted of two catechin monomers (HRESIMS [M+H]+ m/z 291.0864-291.0872 (ppm error; 0.2924 ~ 3.0408), calcd. for $C_{15}H_{15}O_6$, 291.0863), five dimers (HRESIMS [M+H]+ m/z 579.1495 - 579.1516 (ppm error; -0.3508 ~ -7.8446), calcd. for $C_{30}H_{27}O_{12}$, 579.1503), five trimmers (HRESIMS [M+H]+ m/z 867.2108-867.2157 (ppm error; -2.6423 ~ 3.0079), calcd. for $C_{45}H_{39}O_{18}$, 867.2136), and a tetramer (HRESIMS [M+H]+ m/z 1155.2746 (ppm error; -1.6271), calcd. for $C_{60}H_{51}O_{24}$, 1155.2770) (data not shown). Representative selected-ion monitoring chromatograms of proanthocyanidin standard, control cell extract, and proanthocyanidin-treated cell extract are shown in Fig. 10. In the control cell extract, no peaks related to the proanthocyanidin standard were observed. Meanwhile, two peaks corresponding to catechin monomers (m/z = 291.0852 and 291.0860) at the retention times (RTs) of 13.0 min and 14.9 min and two peaks corresponding to catechin dimers (m/z = 579.1499 and 579.1504) at the RTs of 12.1 min and 13.6 min were observed. The level of peaks are summarized in Table 1, showing that the monomer level was almost twice as much as the dimer level.

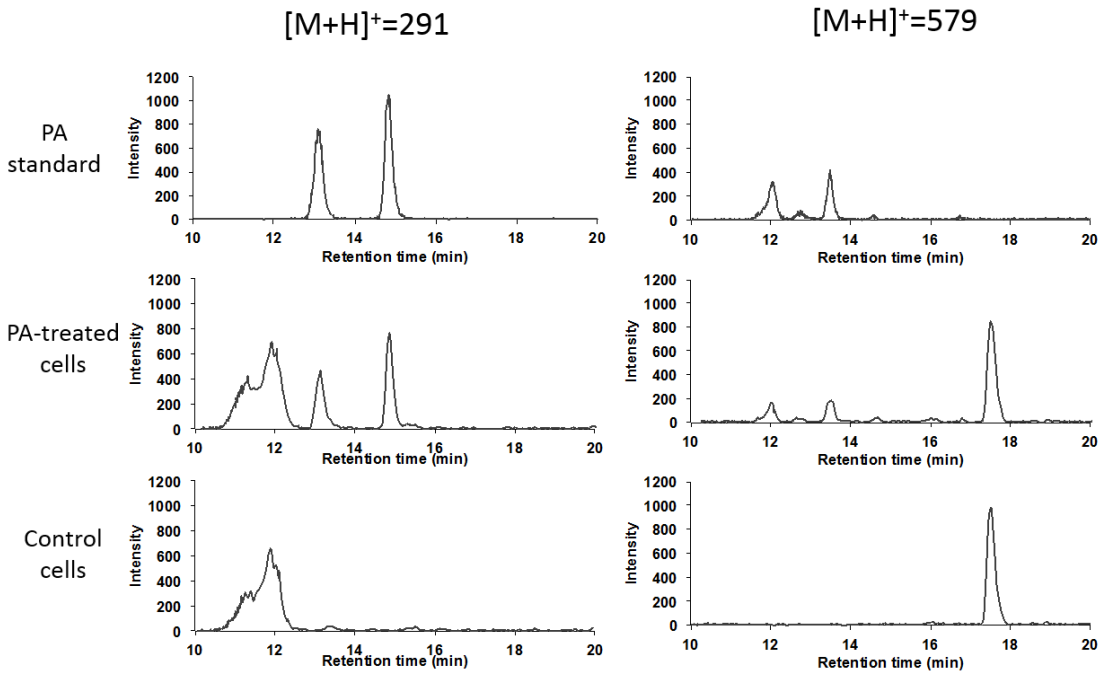

Figure 10

Representative selected-ion monitoring chromatograms of proanthocyanidin standard, control cell extract, and proanthocyanidin-treated cell extract

Five microliter of proanthocyanidin standard (0.02 μg/ml) was subjected to the LC/MS analysis.

PA stands for proanthocyanidin.

Table 1 The level of catechin monomers and dimers obtained from the proanthocyanidin-treated cell extract

Retention time (min)	Molecular formula	Peak (counts)
13.04	C15H14O6 (catechin monomer)	3.16±0.20
14.88	C15H14O6 (catechin monomer)	4.43±0.30
12.08	C30H28O12 (catechin dimer)	1.54±0.25
13.55	C30H28O12 (catechin dimer)	1.76±0.17

Peak count is expressed as the mean±standard deviation (n=3)

Discussion

Based on the hypothesis that photo-irradiated polyphenols could accelerate wound healing through ROS formation, the effect of pretreatment with proanthocyanidin on the mouse fibroblast proliferation was examined with or without photo-irradiation. As a result, pretreatment with proanthocyanidin for a short-time as 1 min significantly accelerated the proliferative response of the cells irrespective of with or without photo-irradiation. Since previous report showed that ROS such as H_2O_2 and hydroxyl radical were formed when proanthocyanidin was photo-irradiated [9] and accelerated proliferation by proanthocyanidin was achieved without photo-irradiation in the present study, extracellular ROS seem to be less likely involved in the accelerated proliferation of the cells. Since the MTT assay is based on an oxidation-reduction reaction, anti-oxidative polyphenols might affect the reduction of MTT to form formazan. Thus, the NR assay was also applied to determine the cell viability in this experiment with proanthocyanidin, and the result clearly showed that proanthocyanidin accelerated the proliferative response. From these, it is considered that incorporated proanthocyanidin into the cells within a short time would exert the effect. To further confirm if other polyphenols possess similar property to that of proanthocyanidin, effect of pretreatment with several phenolic compounds and catechins for 1 min on the proliferative response was examined. As a result, like proanthocyanidin, caffeic acid, chlorogenic acid, and (+)-catechin accelerated the proliferation but gallic acid, ECG, EGC, and EGCG failed to accelerate the proliferation within a range of concentrations applied (0.032 to 0.5 mg/ml). Since proanthocyanidin contains a monomer and a polymer of flavan-3-ol, such as (+)-catechin, EC, and ECG, catechins tested were expected to accelerate the proliferation. However, ECG, EGC, and EGCG failed to accelerate the proliferation at the concentrations tested.

At the moment, although it cannot be stated that ECG, EGC, and EGCG do not possess the ability to accelerate the proliferation, at least their ability seems to be low at weight base as compared to that of proanthocyanidin, caffeic acid, chlorogenic acid, and (+)-catechin even though ECG, EGC, and EGCG could accelerate the proliferative response. One of the possibilities that these catechins failed to accelerate the cell proliferation is the galloyl group in their chemical structures. Since galloyl group possesses three hydroxyl groups, attenuated hydrophobicity might be responsible for reduced cell permeability of these catechins. Regarding the putative active site of the polyphenols exerting accelerating effect on cell proliferation, it might be a moiety containing catechol because all of the polyphenols that exerted the effect in the present study possess catechol moiety in their structures. As structure-activity relations and physicochemical properties of various kinds of polyphenolic compounds would give us helpful information, we should conduct such study in the near future.

According to the result of exposure time experiment using proanthocyanidin and caffeic acid, 0.5-min pretreatment would be enough to accelerate the proliferative response of the cells. In the case of caffeic acid, however, 32-min pretreatment resulted in the less proliferative response than that in the control, probably due to its cytotoxic effect. Indeed, a previous study showed that caffeic acid showed severe cytotoxicity in mouse lung fibroblasts upon 72 h contact [20]. If incorporated active ingredient such as proanthocyanidin for such a short time as 1 min accelerates the proliferation response, a bioassay was conducted by utilizing antioxidant potential of proanthocyanidin [21]. That is, oxidative stress was induced to the cells pretreated with proanthocyanidin. Once H_2O_2 was added to the cells, intracellular oxidative stress as expressed by DCF equivalent increased, and this increase was significantly suppressed by the pretreatment with

proanthocyanidin, indicating that intracellular oxidative stress was significantly suppressed in the cells pretreated with proanthocyanidin (1 mg/ml) for 1 min. Thus, it is suggested that incorporated proanthocyanidin into the cells exerted antioxidant effect on the oxidative stress induced by H_2O_2. Since it was reported that polyphenols including proanthocyanidin could scavenge H_2O_2 in some degree [22-25] and it was confirmed that proanthocyanidin could directly scavenge H_2O_2 (data not shown) as reported by the previous study [25], the suppressed oxidative stress would be caused by scavenging effect of proanthocyanidin on H_2O_2. Besides H_2O_2, hydroxyl radical might be intracellularly generated by a Fenton reaction in which H_2O_2 is reduced to hydroxyl radical in the presence of intracellular transition metals. Indeed DCFH is oxidized by not only H_2O_2 but hydroxyl radical [26]. In addition, if the proanthocyanidin had adsorbed to the cell membrane and the adsorbed proanthocyanidin had then increased cell permeability, the intracellular DCF would have leaked from the cells, resulting in a reduced level of intracellular DCF. Thus, this point was checked by conducting the following experiment. That is, the cells were firstly treated with DCFH-DA followed by H_2O_2-load. Then the cells were exposed to saline or proanthocyanidin for 1 min followed by replacement with the fresh medium without serum supplementation. Immediately after and 20 min after the replacement, DCF level in the culture supernatant was fluorospectrometrically determined (data not shown). As a result, DCF level increased significantly in the control culture supernatant, indicating that intracellular DCF somewhat leaked. However, the DCF level in the proanthocyanidin-treated culture supernatant did not show such a significant increase, likely reflecting low level of intracellular DCF. Thus, the reduced DCF by the proanthocyanidin-pretreatment would not be induced by the leakage of intracellular DCF. As such, even though proanthocyanidin in part would be adsorbed to

the cell membrane, it was concluded that proanthocyanidin incorporated into the cells prevented the oxidation of DCFH caused by H_2O_2-load. Incorporation (or in part adsorption) of proanthocyanidin component such as catechin monomers and dimers into the cells within 1 min was also confirmed by the HRESIMS analysis. Since catechin monomers were detected by the HRESIMS analysis, it was further examined if pretreatment with (+)-catechin exerts antioxidant effect on the oxidative stress induced by H_2O_2 as is the case with proanthocyanidin as shown in Fig. 9. As expected, the increase in DCF induced by H_2O_2 was significantly suppressed (data not shown). These evidences suggest that active ingredient such as proanthocyanidin components and some kinds of polyphenols could be incorporated into the cells in such a short time as 1 min, and resultant incorporated active ingredient could accelerate the proliferative response of the cells. Molecular base mechanism by which proliferative response of the cells pretreated with proanthocyanidin, caffeic acid, chlorogenic acid, and (+)-catechin is accelerated will be further examined in the near future.

Since fibroblast proliferation is one of the pivotal events for wound healing [13-15], it is expected that short time treatment of wounded lesion with active polyphenols such as proanthocyanidin would accelerate healing process. Considering that the accelerative effect on the cell proliferation could be produced even under the photo-irradiation and photo-irradiated polyphenols could exert potent antibacterial effect, active polyphenols found in the present study would be novel antiseptic wound healing agents in combination with photo-irradiation. The concentration of proanthocyanidin used in the present study was similar to that in the study on the bactericidal effect of photo-irradiated proanthocyanidin [12], but the treatment time with photo-irradiated proanthocyanidin to achieve 3-log reduction of *Staphylococcus aureus* was 10 min that was relatively longer

than the treatment time in the present study. Thus, antiseptic wound healing effect of the photo-irradiated active polyphenols will be further confirmed by using an *in vivo* superficial wound infection model especially in terms of treatment time [27, 28]. If the antiseptic wound healing effect of the photo-irradiated active polyphenols is confirmed, the technique would be applicable to the treatment for damaged oral tissues especially induced by infectious agents. The goal of the present study is to develop such a novel treatment method.

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